REMARKS/ARGUMENTS

With this amendment, claims 15-17, 20 and 21 are pending. For convenience, the Examiner's rejections are addressed in the order presented in an October 2, 2003, Office Action.

I. Status of the claims

Claims 15-17 and 20-21 are amended to correct antecedent basis and other obvious grammatical errors. These amendments are not limiting amendments and add no new matter.

II. Objections to the abstract and specification

The abstract is objected to for allegedly lacking proper language, format, and content. In order to expedite prosecution, Applicants have amended the abstract to more clearly describe the invention.

The specification at page 8 is objected to because references to Figure numbers were inadvertently omitted. In order to expedite prosecution, three paragraphs on page 8 are amended to include reference to Figure numbers.

In view of the above amendments, Applicants respectfully request that the objections to the abstract and specification be withdrawn.

III. Rejections under 35 U.S.C. §101

Claims 15-17, 20, and 21 are rejected under 35 U.S.C. §101 for allegedly lacking a disclosed specific and substantial credible utility. The Office Action states that the application provides a description of an R0101 protein and an isolated DNA encoding such protein, but alleges that the specification does not provide the biological role or significance of the protein. Applicants respectfully traverse the rejection. At a minimum, the application asserts that changes in expression levels of R0101 proteins and encoding nucleic acids can be used to diagnose cell cycle associated disorders (which specifically include cancer) or to determine prognosis of such disorders. (Specification at page 41, lines 21-23; and at page 40, lines 23-24.)

As described in detail below, the application provides evidence that R0101 expression levels are indeed upregulated in certain cancers compared to non-cancerous tissue and supporting evidence is also disclosed in a post-filing reference. Because of the utility of the R0101 protein, the application provides utility for methods to screen for bioactive agents that bind to the R0101 protein. Thus, the application provides both an assertion of and evidence of utility of the claimed invention.

The current and previous Office Actions repeatedly allege that neither the specification nor art of record identify even a single disease or disorder that is associated with the claimed R0101 protein. Applicants assert that the specification as filed, in combination with previously submitted post-filing reference (Yu *et al.*, *Oncology* 20:484-489 (2001), resubmitted as Exhibit A), demonstrate that overexpression of R0101 mRNA is associated with cancer, specifically with breast, uterine, cervical, brain, kidney, lung, and esophageal cancers. Applicants further assert that the data shown in Figure 5 of the application and the data shown in Figure 5 of Yu *et al.* are the same. The description of figure 5 of Yu *et al.* states that the blot is an mRNA blot purchased from a commercial source and that the top row is probed with labeled $p15^{PAF}$ (*i.e.*, R0101) nucleic acid and that bottom row was probed with β-actin as a control. Both figures show matched pairs of tumor and normal tissues from a number of tissue types. Samples with elevated levels of $p15^{PAF}$ (*i.e.*, breast, uterine, cervical, brain, kidney, lung, and esophageal cancers) are labeled with asterisks in both figures. Thus, the specification does provide evidence that R0101 is associated with cancer.

With regard to the evidence presented in support of utility in this and the previous response, Applicants respectfully point out that there is no predetermined amount of evidence that must be provided to support utility. MPEP 2107.02.VII. Evidence is sufficient if it leads one of skill in the art to "conclude that the asserted utility is more likely than not true." *Id.* Yu *et al.*, a publication in a peer reviewed journal, demonstrates that one of skill would consider the asserted utility to be more likely than not true. Yu *et al.* contains assertions of the utility of $p15^{PAF}$ (*i.e.*, R0101) as a prognostic indicator for certain cancers at page 487, column 1. *E.g.*, "...elevated levels of $p15^{PAF}$ in tumor tissues may be a useful prognostic parameter for certain types of cancer." Applicants remind the Examiner that an asserted utility does not have to be

established as "true beyond a reasonable doubt." MPEP 2107.02.VII. Instead, as Applicants provide here, all that is required is a reasonable correlation between the asserted use and biological activity. MPEP 2107.03.I.

In view of the above arguments, Applicants respectfully request that the rejections under 35 U.S.C. §101 be withdrawn.

IV. Rejections under 35 U.S.C. §112, first paragraph, enablement

Claims 15-17, and 20-21 are rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter that was not described in the specification as filed in a manner to enable one of skill to make or use the invention. The Office Action asserts that, since the R0101 protein allegedly lacks utility, a method of screening for a bioactive agent that binds to an R0101 protein also lacks utility.

To the extent the rejection applies to the amended claims, Applicants respectfully traverse the rejection. According to the MPEP and to the Federal Circuit, a claim is enabled if the invention can be practiced by one of skill in the art without undue experimentation. Applicants assert that the R0101 protein has utility as argued above and herein, and the specification teaches one of skill how to practice the claimed invention without undue experimentation.

With regard to enablement of use, the Office Action asserts that R0101 is not enabled because it does not correspond to a previously known protein with known activity that lacked sequence information. Applicants respectfully disagree. First, there is no requirement in U.S. code, case law, or regulations that a claimed protein must correspond to a previously known protein with known activity in order to meet the enablement requirement. Second, the specification provides the protein sequence of R0101, the activity of R0101 (*e.g.*, binding to PCNA), and correlates R0101 overexpression with the disease cancer. Thus, the specification fulfills the "how to use" portion of the enablement requirement, without reference to some previous disclosure of the R0101 protein.

The Office Action also alleges that use of R0101 related proteins (*i.e.*, proteins that have at least 95% identity to SEQ ID NO:2 and PCNA binding function) in binding assays is

beyond the skill of those in the art and would require undue experimentation. The use of R0101 related proteins in binding assays is disclosed at page 27, line 32 through page 30, line 32 of the specification. The disclosed methods can be used to determine binding to R0101, as they can be used with any protein. The Examiner has not provided any evidence or arguments to suggest that R0101 or related proteins would not be capable of being used in the binding methods disclosed in the application; *e.g.*, being bound to commonly used supports, *e.g.*, microtiter plates, arrays, membranes or beads; being labeled for use in binding assays; or being used in a competitive binding assay. Thus, one of skill, on reading the specification, would be able to practice the binding assays without undue experimentation.

The Office Action also alleges that there is no nexus between overexpression of R0101 and cancer because the data in the application could be interpreted to mean that R0101 is a metabolically active protein and that R0101 overexpression might not be a direct cause of the malignancy. Applicants respectfully disagree. The observation that R0101 is overexpressed in some cancers is sufficient to provide a nexus between R0101 overexpression and cancer. No causal relationship is required for a protein or mRNA to act as a cancer diagnostic or prognostic indicator. For example, measurement of prostate specific antigen (PSA) is widely used as a diagnostic tool to assist in prostate cancer detection. PSA is a secreted protein with serine protease activity that causes liquefaction of seminal coagulum, a biological function that is not obviously associated with cellular proliferation. (Osterling *et al. Cancer of the Prostate*, in Cancer Principles & Practice of Oncology, De Vita *et al.*, eds. (1997)). No direct causal role of PSA in prostate cancer has been demonstrated or required in order for the protein to act as a diagnostic indicator of the disease. Thus, based on the evidence in the application, the assertion that overexpression of R0101 is associated with cancer is not mere speculation and, in fact, supports both the utility and enablement of the claimed invention.

In view of the above arguments, Applicants respectfully request that the rejection for alleged lack of enablement be withdrawn.

V. Rejections under 35 U.S.C. §112, first paragraph, written description

Claims 15-17, and 20-21 are rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter that was not described in the specification as filed. According to the Office Action, the description is not adequate because allegedly 1) no description is given of experimentation of combining the R0101 protein with a candidate bioactive agent and 2) the generic claim lacks a representative number of species for support. To the extent the rejection applies to the amended claims, Applicants respectfully traverse.

According to the MPEP and the Federal Circuit, " a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention." MPEP 2163.I. Possession can be shown in a variety of ways, but reduction to practice is not required.

> Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. *Citations omitted, id.*

The Office Action alleges that a description of experimentation of combining R0101 with candidate bioactive agents to determine binding, *i.e.*, reduction to practice, is required for possession. However, reduction to practice is not required for possession, and moreover, identifying characteristics of the claimed methods, *e.g.*, binding assays, were sufficiently described in the specification. The application provides ample description of binding assays for use in the claimed methods, *e.g.*, page 27, line 32 through page 30, line 32 of the specification. The Office Action alleges that a binding event has not been adequately defined. However, determination of binding is defined is defined in the specification, *e.g.*, direct binding at page 28, lines 29-33; competitive binding at page 29, line10 through page 30, line 15. The specification also describes controls used in binding assays, *e.g.*, at page 30, lies 21-26. Based on this disclosure of well known methods, one of skill would conclude that the inventors were in possession of the invention at the time of filing.

The specification also alleges that the genus of R0101 related proteins used in the claimed methods lacks disclosure of a sufficient number of species and is thus not adequately described. Applicants respectfully traverse this rejection. The claimed methods recite recombinant proteins that share at least only 95% identity to SEQ ID NO:2 and that have the function of binding to PCNA. Thus, only 6 amino acids can be changed in the 111 amino acid R0101 sequence (*i.e.*, SEQ ID NO:2) and fall within the scope of the claims. The specification further includes the amino acid sequence of R0101 and points out a conserved amino acid sequence that is required for PCNA binding. *See, e.g.*, Figure 2A. Thus, the structure of the protein is described. In addition, according to the MPEP, binding affinities and binding specificities are also identifying characteristics of a protein. (*I.e.*, "For some biomolecules, examples of identifying characteristics include a sequence, structure, binding affinity, binding specificity, molecular weight, and length." MPEP 2163). The application provides description of and examples of R0101 binding to PCNA. Thus, the application provides the required description of the genus of R0101 proteins recited in the claims.

In view of the above arguments, Applicants respectfully request that the rejection for alleged lack of written description be withdrawn.

VI. Rejections under 35 U.S.C. §112, second paragraph

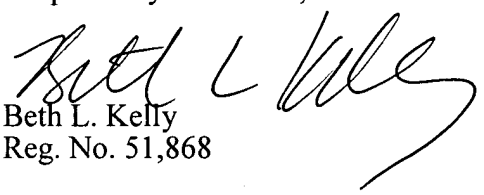
Claims 15-17 and 20-21 are rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. In order to expedite prosecution, claims 15, 17, and 20 are amended to correct antecedent basis. In view of the amendments, Applicants respectfully request that the rejection under 35 U.S.C. §112, second paragraph be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at 415-576-0200.

Respectfully submitted,



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p15^{PAF}, a novel PCNA associated factor with increased expression in tumor tissues

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Proliferating cell nuclear antigen (PCNA) is an essential protein in both DNA replication and DNA damage repair. A novel 15 kD protein, p15^{PAF}, was identified as a *P*CNA-*a*ssociated *f*actor in a yeast two-hybrid screen using PCNA as the bait. p15^{PAF} is localized primarily in the nucleus. p15^{PAF} shares the conserved PCNA binding motif with several other PCNA binding proteins including CDK inhibitor p21. Overexpression of p15^{PAF} competes with p21-PCNA binding. Mutation of this motif in p15^{PAF} abolished its PCNA-binding activity. Notably, p15^{PAF} expression in several types of tumor tissues was significantly increased, especially in esophageal tumors. Like PCNA, p15^{PAF} may possess prognostic significance in a broad array of human cancers. *Oncogene* (2001) **20,** 484-489.

Keywords: PCNA; p21; tumor; PIP box

Introduction

The proliferating cell nuclear antigen, PCNA, is an auxiliary factor for DNA polymerase processivity. PCNA interacts with numerous DNA replication/repair enzymes, such as DNA polymerase delta and epsilon, replication factor C (RF-C p140), DNA ligase 1, replication endonuclease FEN-1, Uracil-DNA glycosylase 2, MLH1, MSH2 and the DNA repair endonuclease, XPG (Chuang *et al.*, 1997; Loor *et al.*, 1997; Umar *et al.*, 1996). Most of these enzymes do not recognize DNA sequences with high specificity. PCNA binds to double strand DNA as a homotrimer and serves as a platform to tether polymerases to the DNA template during DNA synthesis (Kelman and Hurwitz, 1998). PCNA has been shown to interact with DNA (cytosine-5) methyltransferase (MCMT) in post-replication DNA synthesis. MCMT methylation of newly synthesized DNA regulates chromatin organization and gene expression.

Several non-enzymatic cell cycle regulators, such as CDK inhibitors p21$^{CIP/WAF1}$ (Xiong *et al.*, 1992; Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994), p57^{Kip2} (Watanabe *et al.*, 1998), and Gadd45 (Smith *et al.*, 1994; Hall *et al.*, 1995; Sanchez and Elledge, 1995), also bind to PCNA. p21 and p57 both contain two separate binding activities: a CDK-cyclin binding domain at the N-terminus and a C-terminally located PCNA binding site (Watanabe *et al.*, 1998; Luo *et al.*, 1995; Nakanishi *et al.*, 1995), both of which are capable of inhibiting cell cycle progression. Mutagenesis and p21-PCNA crystal structure analysis have identified a short amino acid motif (^{144}QTSMTDFY^{151} in human p21 and ^{268}SGPLISDFF^{276} in human p57) critical for binding to PCNA. In particular, the hydrophobic Met/Leu and Phe residue (underlined), are required for p21 and p57 to bind PCNA at high affinities (Watanabe *et al.*, 1998; Warbrick *et al.*, 1995; Gulbis *et al.*, 1996). In addition to these two CDK inhibitors, a similar conserved PCNA binding motif (also named PIP-box) containing QXXL/I/MXXF/Y (Warbrick, 1998), is also found in several other PCNA interacting proteins, including xeroderma pigmentosum group G (XPG; Ludwig *et al.*, 1997), flap endonuclease (FEN-1; Li *et al.*, 1995) and DNA-(cytosine-5) methyltransferase (MCMT; Chuang *et al.*, 1997). The p21-PCNA association does not affect the overall structure of PCNA or the PCNA-DNA association (Gulbis *et al.*, 1996). Instead, p21 is capable of competing with these PCNA-binding proteins, potentially preventing PCNA from binding to DNA polymerase and other replication factors. In this study, we identified a novel 15 kD PCNA-associated factor, p15^{PAF}. p15^{PAF} contains the conserved PCNA binding motif in which a mutation disrupts PAF-PCNA binding. Unlike p21 and p57, overexpression of p15^{PAF} does not inhibit cell cycle progression. Of potential significance, the expression of p15^{PAF} is substantially elevated in several types of tumors. We suggest that p15^{PAF} may be a new member of PCNA associated cell proliferation family of regulators.

Results

Identification of p15^{PAF}

A large number of proteins have been identified through binding to PCNA. To clone less abundant

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EXHIBIT A

PCNA associated proteins, we constructed a yeast two-hybrid cDNA library from mixed human tissues of adult whole brain, fetal whole brain, thalamus, and hippocampus at 1:1:1:1 ratio instead of using only whole brain mRNA. This library contains 36 million independent clones. Twenty million transformants were screened. A total of 73 positive clones were identified, including three known PCNA binding proteins (p21, FEN-1, and Uracil-DNA glycosylase 2), validating the authenticity and efficiency of the screen. Five of the 73 PCNA-interacting clones contained cDNA inserts corresponding to the expressed sequence tag (EST) clone KIAA0101 deposited in GenBank (Nagase *et al.*, 1995) (Figure 1a). These five positive clones all contain the PCNA binding motif and encode the entire coding region of KIAA0101. KIAA0101 (D14657) was conceptually translated but is functionally uncharacterized. Due to its PCNA-binding activity, we have therefore named KIAA0101 as PAF (PCNA associated factor). Notably, PAF contains sequence closely related to the PCNA-binding motif present in other PCNA-interacting proteins (Warbrick, 1998) (Figure 1b). In particular, three highly conserved residues, Gln, Met/Ile/Leu and Phe, are all conserved in PAF, suggesting that PAF may bind to PCNA in a similar manner as other PCNA binding proteins.

p15PAF binds to PCNA in mammalian cells

Conceptual translation of PAF predicts a 15 kD protein (p15PAF), which was confirmed by transient transfection and immunoprecipitation (Figure 2). To confirm the binding of p15PAF with PCNA in mammalian cells, HA-tagged p15PAF was recloned from the positive yeast-two-hybrid clone into a CMV promoter expression vector (pYCI) by PCR and was transfected into 293 cells. Figure 2 shows HA-tagged p15PAF co-immunoprecipitated with endogenous PCNA (Figure 2a,b). Mutation of two conserved residues in the putative PCNA binding motif (I65A, F68A) of the PIP-box, completely disrupted the binding of p15PAF to PCNA (Figure 2c, lane 3). The lower panel of Figure 2c shows that both the wild type and the mutant p15PAF were expressed at similar protein levels. This result demonstrated that the PIP-box is required for p15PAF binding to PCNA.

p15PAF competes with p21 for binding to PCNA

p21 has been shown to compete with DNA polymerase and FEN-1 for binding to PCNA. Since both p15PAF and p21 share the same PCNA-binding motif, we tested whether p15PAF can compete with p21 for binding to PCNA. HA-tagged p15PAF was co-transfected with Flag-tagged p21 into 293 cells, and p15PAF binding to PCNA was diminished in the presence of increasing concentration of p21 (Figure 2b, upper panels). When Flag-tagged p21 was co-transfected with GFP or HA-tagged p15PAF into 293 cells, p21's binding to PCNA was also inhibited by increasing the amount of p15PAF (Figure 2b, lower panels). Results from HA-tagged p15PAF co-transfection are not shown. Since only very high levels of p15PAF (6:1 ratio) are able to compete with p21 for PCNA association, it is speculated that the affinity of p15PAF to PCNA may be weaker than that p21, although endogenous levels of p21 and p15PAF were not measured in transfection experiments.

Tissue-specific expression and nuclear localization of PAF

PAF gene was expressed as a 1.1 kb message that accumulated in liver, pancreas and planceta at a high levels (Figure 3). PAF expression was not detected in heart or whole adult brain, despite the initial isolation from a brain cDNA library. To determine the subcellular localization, we fused full length PAF to the C-terminal of GFP and expressed PAF–GFP protein in Hela cells by transient transfection. As shown in Figure 4, the majority of green fluorescence was observed in the nucleus, although some cytoplasmic distribution can also be seen. Hence, PAF is mainly localized to the nucleus.

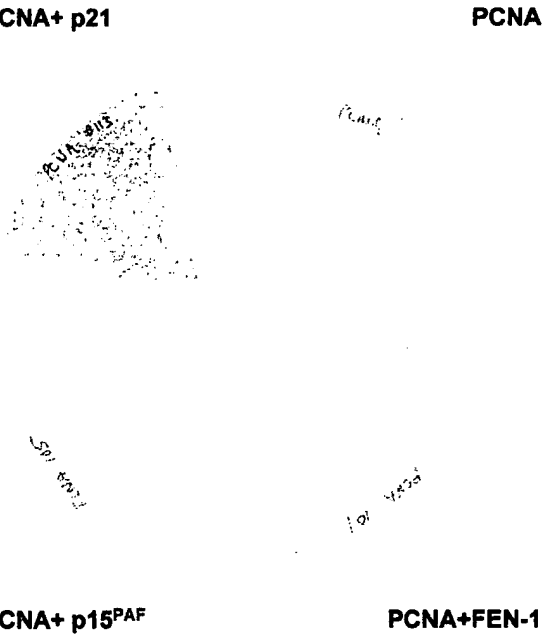
p15PAF expression is increased in tumor tissues

PCNA expression has been correlated to tumor progression and is commonly used as a molecular marker for detecting hyperplastic cell growth. Increased p21 expression has also been seen in several types of malignant tumors (Baekelandt *et al.*, 1999; Barboule *et al.*, 1998), and a higher level of p21 has been correlated with higher 5-year survival rate (Kuwahara *et al.*, 1999; Natsugoe *et al.*, 1999; Ropponen *et al.*, 1999). To evaluate p15PAF's potential function in tumor development, p15PAF expression level was measured using tumor blots (Invitrogen). As shown in Figure 5, the p15PAF mRNA level was significantly increased in esophageal, breast, uterine, cervix, brain, kidney and lung tumors. This increased mRNA level is especially dramatic in esophageal tumor (>10-fold). Northern analysis also verified that in normal whole adult brain tissue, little or no p15PAF was detected. Little or an undetectable change of p15PAF expression was observed in colorectal cancer or pancreas tumor.

Discussion

Both PCNA and p21 have been shown to interact with a large number of cellular proteins involved in DNA replication/repair and cell cycle control. Most of the non-enzymatic PCNA binding proteins identified, such as p21 and p57, inhibit DNA synthesis and cell cycle progression. Since p15PAF is able to compete with p21 for binding to PCNA, it is plausible to speculate that over-expression of p15PAF in tumor tissues may be advantageous for tumor cell proliferation. However, co-transfection of p15PAF with p21 expression plasmid into Saos-2 and 293 cells did not show a significant change in p21 induced cell cycle arrest. In addition, PCNA has more than one p21/p15PAF binding site and may bind to both p15PAF and p21

a



b

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MVRTKADSVPGTYRKVVAARAPRKVLGSSTSATNSTSVSSRKAENKYAGG  50
NPVCVRPTPKWQKGIGEFFRLSPKDSEKENQIPEEAGSSGLGKAKRKACP 100
LQPDHTNDEKE  111
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c

p15PAF	(62-69)	QKGIGEFF
P21	(142-149)	QTSMTDFY
P57	(269-276)	GPLISDFF
XPG	(991-999)	QLRIDSFF
FEN1	(333-340)	QGRLDDFF
MCMT	(164-171)	QTTITSHF

Figure 1 p15PAF was identified in a yeast two-hybrid screen using PCNA as the bait. (**a**) Cloning of p15PAF by binding to PCNA Yeast Y190 cells were simultaneously transformed with a plasmid expressing a GAL4bd fusion protein and a plasmid expressing a GAL4ad fusion protein as indicated. Cells were streaked on non-selective medium with histidine (−Leu, −Trp), selective medium without histidine (−Leu, −Trp, −His) and selective medium without histidine but containing 5 mM 3-amino-1,2,3-triazole (−Leu, −Trp, −His, 3-AT). Staining for β-galactosidase expression, activated from an independent GAL4 responsive promoter, is shown lower right panel (denoted by β-gal). The C-terminal domain of p21 possesses a trans-activating activity (self-activation) when expressed as fusion protein with the GAL4 DNA binding domain. (**b**) Amino acid sequence of human PAF/KIAA0101. The conserved PCNA binding motif is underlined, also known as PIP-box. (**c**) Comparison of PCNA binding motif. The number in parenthesis indicate the position of PCNA-binding motif in the respective proteins

at the same time. Without knowing the relative endogenous protein level of p15PAF and p21, it is difficult to determine the relative relationship between p15PAF and p21. Under physiological conditions, p15PAF may not be able to compete with p21 for PCNA binding. p21 binds to PCNA to disrupt DNA replication/repair machinery and to inhibit cell proliferation. However, in transient transfection experiments using Hela, Saos-2, and 293 cells, we were not able to detect any cell cycle inhibition by the overexpression of p15PAF (data not shown). An *in vitro* DNA synthesis assay may be needed to reveal the detailed mechanism of p15PAF function.

Since the mRNA level of p15PAF is dramatically increased in many types of tumors, we amplified (by PCR) and sequenced p15PAF from kidney, liver, lung and esophageal tumors. No mutation was found in p15PAF

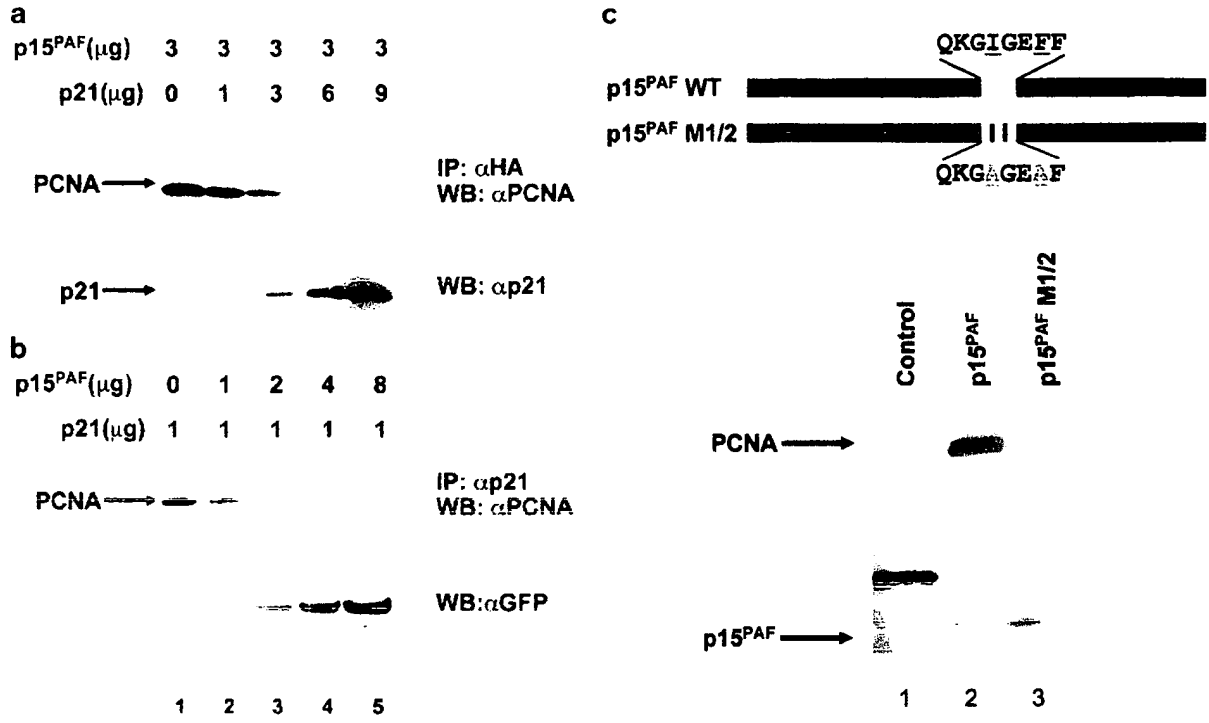

Figure 2 p15^{PAF} competes with p21 for binding to PCNA in mammalian cells. (**a**) p21 competes with p15^{PAF} for binding to endogenous PCNA. 3 μg of HA-tagged p15^{PAF} expression plasmid (pYCI) was co-transfected with 0–9 μg of p21 expression plasmid into 293 cells (Phoenix A cells). Cell lysate was immunoprecipitated by anti-HA antibody and subsequently blotted with anti-PCNA antibody. The amount of p21 protein in a control experiment is shown in the lower part of the panel. (**b**) p15^{PAF} competes with p21 for binding to endogenous PCNA. 1 μg of p21 expression plasmid was co-transfected with 0–8 μg GFP-tagged p15^{PAF} expression plasmid. Cell lysate was immunoprecipitated by anti-p21 antibody and subsequently blotted with anti-PCNA antibody. In the control experiment shown in the lower part of the panel, the amount of GFP-tagged p15^{PAF} is shown by blotting with an anti-GFP antibody. (**c**) Mutation of PIP-box disrupts binding of p15^{PAF} to PCNA. 3 μg of HA-tagged p15^{PAF} wt/mutant plasmid was transfected into 293 cells (Phoenix A cells). Cell lysate was immunoprecipitated with anti-HA antibody and blotted with anti-PCNA antibody (upper panel). Lower panel shows protein amount of p15^{PAF}. Control lane is a non-specific HA-tagged protein

(data not shown). This result excludes the possibility that a dysfunctional copy of p15^{PAF} is over-expressed in tumor tissues. Increased expression of p15^{PAF} in many tumor tissues is not surprising. Expression of PCNA and PCNA-associated proteins such as p21 is also increased in several types of cancers including ovarian cancer and breast cancer. The inhibitory effect of p21 may be overcome by coordinated accumulation of PCNA, cyclinD1 and CDKs (Russell *et al.*, 1999). Similarly, the impact of increased expression of p15^{PAF} in tumor tissues may be neutralized by elevated expression of its binding partners, such as PCNA. However, elevated levels of p15^{PAF} in tumor tissues may be a useful prognostic parameter for certain types of cancer. In esophageal squamous cell carcinoma patients, for example, the 5-year survival rate of p21 positive patients is better than that of p21 negative patients. In other types of cancer, p21's value as an independent prognostic marker is not conclusive. Since p15^{PAF} expression in esophageal tumor is dramatically elevated compared with normal tissue, the possibility exists that p15^{PAF} levels could be used to predict clinical prognosis for esophageal cancer patients.

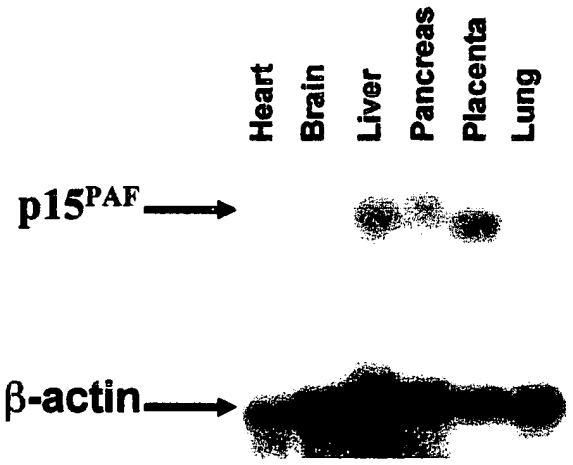

Figure 3 Northern blot of p15^{PAF} tissue expression pattern. Northern blot was purchased from Invitrogen (D1801-08). The band of p15^{PAF} is indicated by arrow. β-actin is used as control. The DNA probe of p15^{PAF} was ^{32}P labeled using an Ambion kit (#1455). Kodak X-ray film was exposed for 24 h after hybridization

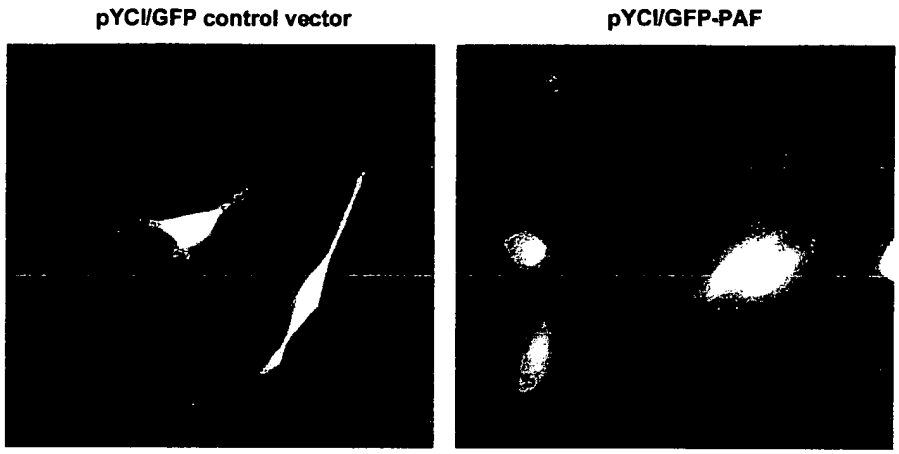

Figure 4 p15PAF is localized in the nucleus. GFP-tagged control vector and PAF–GFP were transiently transfected into Hela cells. Twenty-four hours after transfection, fluorescence was recorded using a Nikon TE300 fluorescence microscope

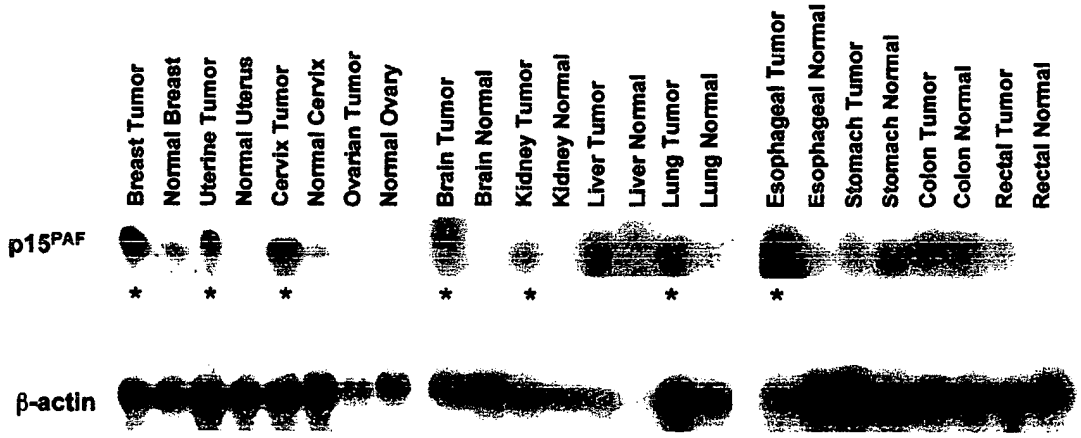

Figure 5 p15PAF is highly expressed in some tumor tissues. Human tumor tissue panel blots were purchased from Invitrogen (D3100-01, D3200-01, D3400-01). β-actin probe was used as control. ^{32}P-labeled p15PAF probe was used and the X-ray film was exposed for 48 h. Tumor tissues with elevated level of p15PAF, such as breast, uterine tissue, cervix, brain, kidney, lung and esophageal tumors are labeled by * underneath (upper panel). The p15PAF band in brain tumor tissue was diffuse in two different tumor blots

Materials and methods

Yeast two-hybrid screening

For yeast two-hybrid screening (Fields and Song, 1989), full length PCNA was used as bait to screen a human brain cDNA library constructed from mixed mRNA of whole adult brain, whole fetal brain, hippocampus, and thalamus. Oligo-dT primer was used to make a unidirectional cDNA library and was ligated into *Xho*I–*Eco*RI sites of the pACT2 vector. Random hexamers were used to construct a second bi-directional library and was ligated into the *Eco*RI site of pACT2. The combined cDNA library represents 36 million independent clones. Yeast strain Y190 was used and 20 million transformants were screened on SD-LWH+3AT (45 mM) plates from a single round of screening.

Transfection and immunoprecipitation

The Ca^{2+} phosphate method was used in all transfection experiments. 2×10^6 Phoenix-A (293 T) cells were harvested 24 h after the transfection of HA-p15PAF (5 μg) and lysed in 0.5 ml lysis buffer (50 mM HEPES [7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA). The immunoprecipitated proteins were prepared by incubating the anti-HA monoclonal Ab with the cell lysates. Volumes were adjusted by normalization with expression of Flag-tagged or HA-tagged proteins analysed by Western blot analysis (protein levels before adjustment for IP shown in middle and bottom panels of Figure 2). The anti-HA immunoprecipitated samples were then washed three times with high stringency lysis buffer (1% NP-40 and 1 M NaCl). For each immunoprecipitation following normalization, aliquots of the lysates were incubated with a 1:1 slurry of anti-HA conjugated Sepharose (BAbCO). The Sepharose beads were washed once with 1 ml lysis buffer and three times with high stringency lysis buffer (1 M NaCl, 1% NP-40). The immuno-precipitated proteins or cell lysates were fractionated on a 4–20% gradient SDS–PAGE gel (Novex).

Cell cycle assay

A549 cells were infected with vector or p15PAF retrovirus supernatant which were collected from transfected Phoneix A

retroviral packaging cells. The top 10% of GFP positive A549 cells 48 h post infection were isolated and cell cycle analysis performed with hypotonic PI solution staining by FCS 72 h post infection. The FCS data was analysed by ModFit LT cell cycle analysis software (Verity Software House, ME, USA).

Fluorescence

GFP-fused p15PAF was transfected into 293 (Phoenix) cells. A Nikon TE-300 fluorescence microscope was used to monitor localization in the transfected cells. The UV excitation wavelength is between 420 and 490 nm and the GFP emission is monitored at 520 nm.

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